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Pathogenesis-Related Proteins of Tomato[1]

P-69 AS AN ALKALINE ENDOPROTEINASE

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ABSTRACT

An endoproteinase induced by citrus exocortis viroid has been purified from tomato (*Lycopersicon esculentum* Mill, cv "Rutgers") leaves. The proteinase corresponds to one of the major pathogenesis-related proteins of tomato plants and was designated proteinase P-69 as it has a molecular weight of 69,000 to 70,000. The proteinase was purified in four steps: $(NH_4)_2SO_4$ fractionation, chromatography on Bio-Gel P-60, DEAE-Sepharose chromatography, and casein-Sepharose affinity chromatography. The proteinase had a pH optimum of 8.5 to 9.0 when assayed with either fluorescein thiocarbamoyl derivative (FTC)-casein or FTC-ribulose 1,5-bisphosphate carboxylase/oxygenase as substrates. The proteinase activity was inhibited by pCMB and strongly activated by calcium and magnesium ions as well as by DTT. When analyzed by electrofocusing, the activity showed a pI around 9.0.

The term PR[2] proteins refers to a group of plant encoded proteins whose synthesis is induced when plants react against infections by viroids, viruses, and several other pathogens as well as stress situations such as those produced by chemical treatments, plasmolysis, and even natural senescence of the plant (29).

Infection of tomato plants by CEV results in a remarkable increase of 10 PR proteins whose synthesis seems to be mediated by ethylene (13).

Although considerable information has been obtained regarding the appearance, properties, amino acid, and nucleotide sequences, and regulation of their synthesis, no specific function or biological activity has been assigned to PR proteins aside from their involvement in protection phenomena (4, 7, 8, 15, 16, 19, 20, 29).

In this paper, we show that one of the major PR proteins induced by CEV in tomato plants, previously reported as P-69 (13), is an endoproteinase. We also speculate about the possible role of P-69 in the infected tissue.

MATERIALS AND METHODS

Plant Material. Tomato plants (*Lycopersicon esculentum* Mill, cv "Rutgers") were grown from seeds in a greenhouse at 20 to 30°C. Inoculation of tomato plants with CEV was performed as described previously (27).

Assay of Proteolytic Activity. The reaction mixture (total volume of 150 μl) contained 10 μl of enzyme (either purified or in crude extracts obtained at pH 2.8 as described below), 130 μl assay buffer, and 10 μl of 0.5% (w/v) FTC-casein. The assay buffer was either 150 mM glycine/NaOH (pH 9.0), 2 mM $CaCl_2$, 0.5 mM DTT, or 150 mM Tris-HCl (pH 9.0), 2 mM $CaCl_2$, 0.5 mM DTT. FTC-casein was prepared from casein and FITC as described (28).

After thorough mixing, the solution was incubated at 37°C for 1 h. The reaction was stopped by addition of 150 μl of 10% (w/v) TCA. The tubes were allowed to stand on ice for 15 min, and the TCA-insoluble material was sedimented by centrifugation for 10 min in a Beckman microfuge. A 60 μl aliquot of the supernatant was diluted to 3 ml with 500 mM Tris-HCl (pH 8.5). Fluorescence was determined using an excitation wavelength of 490 nm and an emission wavelength of 525 nm in a Perkin-Elmer Fluorescence Spectrophotometer.

One unit of FITC-casein-degrading activity is defined as the enzyme needed to produce 1 unit of fluorescence increase under the standard assay conditions. When needed, proteolytic activity was assayed with tomato RuBPCase, purified from tomato leaves as described (26) and labeled with FITC as described for casein, using chromatography on PD-10 columns (Pharmacia, Sweden) instead of dialysis to eliminate free FITC.

Electrophoresis. Electrophoretic analysis of proteolytic activity in acrylamide gels containing SDS and co-polymerized fibrinogen was carried out in 14% polyarcylamide slab gels by the method of Hensen and Dowdle (14) with a final fibrinogen concentration of 0.05%. Normal SDS/PAGE was carried out as previously described (5). Gels were stained for protein with Coomassie blue. The mol wt of the markers used were: BSA (66 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (20 kD), and lactalbumin (14 kD).

Protein Determination. Protein content was measured by the method of Bradford (3) with BSA as a standard.

Purification of Viroid-Induced Proteinase Activity. All operations were carried out on ice or at 4°C. Leaf tissue (30 g) bearing symptoms was frozen in liquid nitrogen and thawed and homogenzied with 30 ml of buffer for 1 min at maximum speed in a Polytron homogenizer. The buffer consisted of 84 mM citric acid, 32 mM Na_2HPO_4 (pH 2.8), 15 mM 2-mercaptoethanol. The homogenate was filtered through cheesecloth, clarified by centrifugation at 20,000*g* for 20 min, and the supernatant was adjusted to 30% saturation with solid $(NH_4)_2SO_4$, centrifuged, and the supernatant was adjusted to 75% saturation with solid $(NH_4)_2SO_4$. The resulting pellet was resuspended in 2.5 to 3 ml of buffer A (35 mM Tris-HCl [pH 5.5], 5 mM DTT) and applied to a Bio-Gel P-60 column (2.5 × 75 cm) equilibrated in buffer A. The void volume fractions containing activity were pooled

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[2] Abbreviations: PR, pathogenesis-related; CEV, citrus exocortis viroid; RuBPCase, ribulose 1,5-bisphosphate carboxylase/oxygenase; pCMB, *p*-chloromercuribenzoic acid; PMSF, phenylmethylsulfonyl fluoride; FITC, fluorescein isothiocyanate; FTC, fluorescein thiocarbamoyl derivative; TPCK, tosyl-L-phenylalanine chloromethyl ketone.

and applied to a DEAE-Sepharose CL6B column (2 × 10 cm) equilibrated in buffer A. The void fractions were pooled, concentrated by freeze-drying, and changed to buffer B (100 mM Tris-HCl [pH 8.5], 5 mM $CaCl_2$, 5 mM DTT) b redissolving in 2.5 ml and passing through a PD-10 column (1.2 × 5 cm) (Pharmacia, Sweden). The eluted fraction was divided into two equal fractions and separately applied to a casein-Sepharose column (1.5 × 5 cm) coupled and prepared as described by the manufacturer (Pharmacia, Sweden). The affinity column was washed with buffer B until no A_{280} in the eluate was observed. Proteinase activity was eluted by washing with 100 mM glycine-HCl (pH 2.8), 500 mM NaCl. The fractions containing activity were pooled, dialyzed against buffer A, and stored at −35°C in 10% (v/v) glycerol.

Molecular Weight Determination of Proteinase Activity. The mol wt of the native enzyme was determined by gel fltration on a Sephadex G-200 column (1.5 × 85 cm). The enzyme and mol wt markers were separately applied to the column equilibrated with 25 mM Tris-HCl (pH 7.5), 100 mM NaCl. The mol wt of the markers used were: β-amylase (200 kD), alcohol dehydrogenase (150 kD), albumin (66 kD), carbonic anhydrase (30 kD), and Cyt *c* (12.4). Void volume was determined with blue dextran (2,000 kD).

Inhibitor and pH Studies. The inhibitors were prepared in a 10-fold concentrated form and diluted to final concentrations with the assay buffer. Proteinase was incubated with the inhibitors for 30 min at 4°C before adding substrate.

The pH optimum studies were carried out with 150 mM Tris-HCl, ranging from pH 6 to pH 10.5. The proteinase was incubated 30 min at 4°C before adding either FTC-casein or FTC-RuBPCase as substrates.

Determination of Proteinase Activity in Gel Electrofocusing. A sample of proteinase was applied to a 5% polyacrylamide gel (10 × 5 cm) containing 1% Ampholine (pH 3–10) (LKB) and subjected to electrofocusing at 600 V for 5 h at 4°C. The gel was sliced into 5-mm fractions, and 1 ml of distilled water was added to each fraction, and the slices kept for 1 h at room temperature. Aliquots (25 μl) of each fraction were separately assayed for proteinase activity. After overnight equilibration of slices, the pH gradient was determined with a pH meter.

RuBPCase Degradations. RuBPCase was subjected to controlled proteolysis at 37°C at a protein-to-proteinase P-69 ratio (w/w) of 220. The reaction mixture consisted of 0.22 mg RuBPCase and 1 μg of proteinase P-69 per ml in 150 mM Tris-HCl (pH 8.5), 1 mM DTT, 0.01% (w/v) SDS. At indicated times, the reaction was quenched by diluting an aliquot containing 20 μg of RuBPCase into SDS sample buffer and heating for 5 min at 100°C. Each sample was analyzed by SDS-PAGE. The gels were stained with Coomassie blue and scanned in a Beckman CDS-200 densitometer.

RESULTS

Proteolytic activity in extracts from viroid-infected leaves was significantly different from activity in extracts of healthy (uninfected) leaves when assayed at pH 9.0 (Fig. 1). The extracts from CEV-infected plants exhibit a characteristic increase in proteolytic activity when compared with extracts of healthy plants.

Purification of the Viroid-Induced Proteinase Activity. Table I shows the scheme for purification of tomato proteinase activity. It was purified in four steps: $(NH_4)_2SO_4$ precipitation, gel filtration chromatography on Bio-Gel P-60, in which the activity eluted in the void volume (Fig. 2), ion-exchange chromatography on a DEAE-Sepharose column to which the activity did not bind and eluted with the washing buffer fractions. The last step of the purification consisted of chromatography on a casein-Sepharose column. The activity was eluted by changing the pH and ionic strength of the buffer (Fig. 3). By this procedure, the proteinase

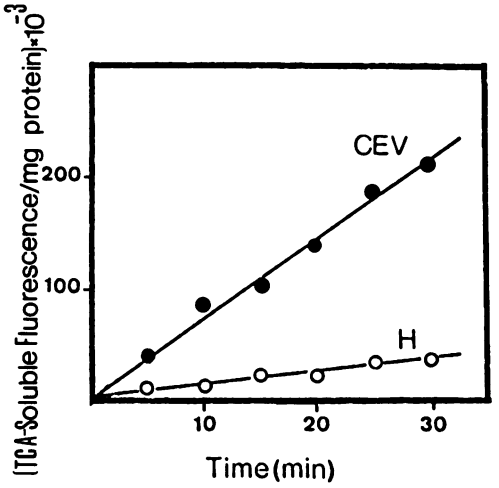

FIG. 1. Time course of FTC-casein degradation by crude extracts obtained from CEV-infected (CEV) and healthy (H) tomato leaves. Degradations were carried out at pH 9.0 for the indicated times as described in "Materials and Methods."

was purified nearly to homogeneity from the acidic crude extract with a recovery greater than 50%.

Electrophoretic Analysis and Molecular Weight Determination. When analyzed by SDS-PAGE, the final enzyme preparation showed a single protein band (Fig. 4). The same purified preparation also acted as a proteinase on SDS-PAGE gels containing fibrinogen (Fig. 4). This protein band showed a mol wt of 69,000 upon normal SDS-PAGE (reducing conditions). The native enzyme exhibited a mol wt of 70,000 as estimated by gel filtration on Sephadex G-200 (Fig. 5). These results suggest that the enzyme is monomeric with a mol wt of 69,000 to 70,000.

Effect of pH. Figure 6 shows the influence of pH on the proteinase P-69 activity with two different substrates. With FTC-casein, the optimal pH for activity was 9.0. When assayed with FTC-RuBPCase (a potential substrate *in vivo*) as substrate, the optimal pH was 8.5. The enzyme showed no activity at acidic pH values (data not shown). Thus, proteinase P-69 is an alkaline proteinase. The activity was soluble and resistant to acidic treatments which render most plant proteins insoluble, thus explaining its enrichment and stability in the starting acidic homogenate.

Effect of Inhibitors. Table II summarizes the effect of various compounds on the activity of tomato proteinase P-69. pCMB exhibited the greatest inhibitory effect. Mercuric ions were also inhibitory. The other compounds had less or no inhibitory effect on the enzyme. Ca^{2+}, Mg^{2+}, and DTT exert in activation effect on the activity.

Determination of pI of Proteinase. Determination of proteinase activity in electrofocusing gels, as explained in "Materials and Methods," indicated that the enzyme has a pI of 8.5 to 9.0 (Fig. 7). This high pI, which is a general characteristic of tomato PRs, makes them capable of entering and being separated in the nondenaturing cathodic electrophoretic system of Reisfeld (13) which is run at pH 4.3. However, these proteins cannot enter into the gels of the nondenaturing anodic system of Davis run at pH 8.3 (29).

Kinetics of P-69 Proteolysis of RuBPCase. In order to elucidate the endoproteolytic behavior of tomato P-69 proteinase, RuBPCase was treated with proteinase P-69 at 37°C at a protein-to-proteinase ratio (w/w) of 220. At the times indicated (Fig. 8) samples of the reaction mixture were analyzed by SDS-PAGE. As the digestion proceeded, the large subunit (L = 55 kD) disappeared and fragments of smaller mol wt (46, 28, 24, 21, 17, and 14 kD, and smaller fragments not resolved in the electro-

Table I. *Purification of Tomato P-69 Proteinase from CEV-Infected Tomato Leaves*

The starting material was 30 g of leaves exhibiting disease symptoms. One unit is defined as the amount of enzyme needed to produce an increase of 1 in acid-soluble fluorescence under the standard assay conditions described in "Materials and Methods."

Purification	Protein	Total Activity	Specific Activity	Yield	Purification
	mg	*units* × 10^{-3}	*units/mg protein* × 10^{-3}	%	*-fold*
Crude extract	22.5	18,000	800	100	
30–70% $(NH_4)_2SO_4$	18.7	17,343	927	94.4	1.2
Bio-Gel P-60	3.81	16,704	4,384	92.8	5.4
DEAE-Sepharose	1.40	13,940	9,957	77.4	12.4
Casein-Sepharose	0.11	9,185	83,500	51	104.3

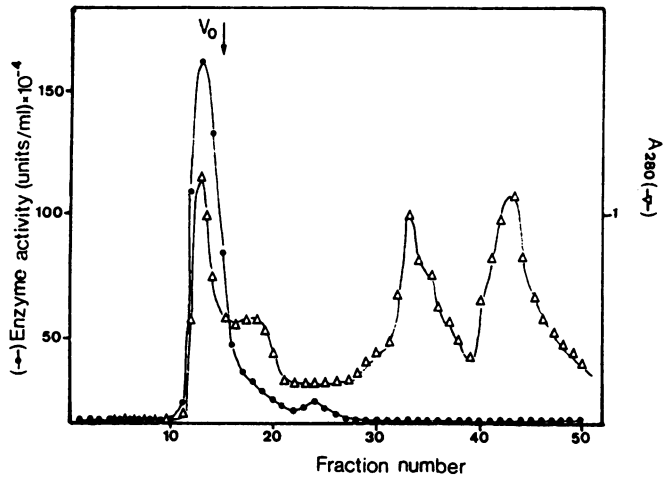

FIG. 2. Gel filtration on Bio-Gel P-60 of the tomato P-69 fraction obtained by 30 to 75% $(NH_4)_2SO_4$ precipitation. The column was eluted at a flow rate of 0.1 ml/min and fractions of 4 ml were collected. Void volume (V_0) is indicated by an arrow.

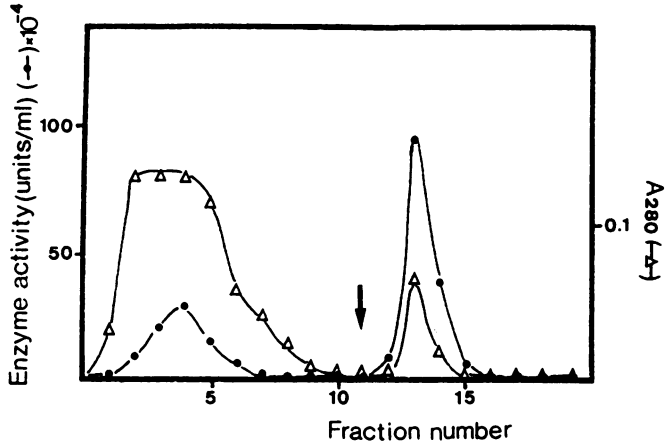

FIG. 3. Casein-Sepharose chromatography of tomato P-69 pooled fractions obtained from DEAE-Sepharose chromatography. The flow rate was 0.25 ml/min and fractions of 3 ml were collected. Arrow denotes the change in pH of the eluting buffer.

phoresis system used) simultaneously appeared. These results indicate that proteinase P-69 is an endoproteinase with high affinity for the RuBPCase large subunit (L). Degradation of the small subunit does not start until 4 h of incubation (data not shown).

DISCUSSION

We show in this paper that infection of tomato plants by CEV significantly enhances an alkaline proteinase activity as compared

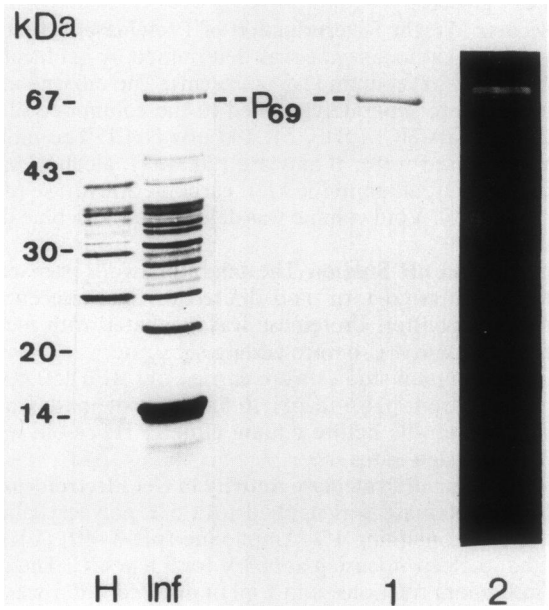

FIG. 4. SDS-PAGE analysis of the final proteinase P-69 preparation. Lane 1 shows the final proteinase preparation obtained after affinity chromatography. Lane 2 shows the same preparation after electrophoresis on SDS-polyacrylamide gels containing fibrinogen and incubation of the gel in Tris-HCl (pH 9.0), 1 mM $CaCl_2$ for 2 h at 37°C and developed as described in the text. H and Inf indicate healthy and CEV-infected acidic leaf extracts. Gels were stained with Coomassie blue. Numbers on the left indicate the migration of mol wt marker proteins in kD. The migration of proteinase P-69 is indicated in the center of the figure.

to healthy controls. The finding that this activity was extracted with the pH 2.8 buffer, which is generally used to extract PR proteins (13), suggested a close relationship between the proteolytic activity and PR proteins. When we analyzed the activity on fibrinogen-containing SDS-PAGE gels, we found that the activity co-migrated with P-69, one of the major PR proteins of tomato plants (13). This proteinase activity copurified with P-69, indicating that both the enzyme activity and PR-P-69 were the same protein. We named this protein proteinase P-69. It exists in a monomeric form as judged by gel filtration and SDS-PAGE, with a mol wt of 69,000 to 70,000. The activity is inhibited by pCMB and mercuric ions, suggesting that it could be classified as a cysteine proteinase. The enzyme is stimulated by calcium and magnesium ions as well as by DTT.

The finding that proteinase P-69 has a pI of 8.5 to 9.0 indicates that this protein is a basic protein, like other tomato PR proteins. All of them can be separated in the native cathodic Reisfeld electrophoretic system (13) but not in the native anodic Davis

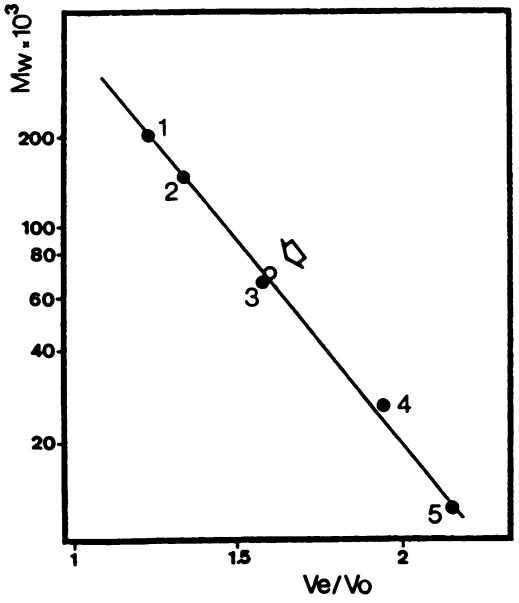

FIG. 5. Mol wt determination of proteolytic activity by gel filtration chromatography on Sephadex G-200. The calibration curve was constructed with proteins of known mol wt (●) as described in "Materials and Methods." Mol wt of proteolytic activity is indicated by an arrow (○).

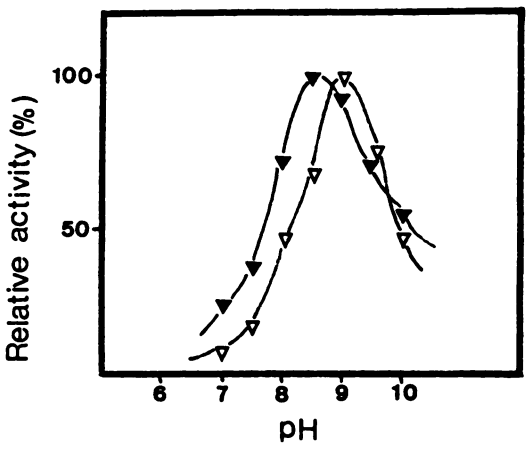

FIG. 6. Effect of pH on the activity of purified tomato P-69 proteinase with different substrates. The buffer used was 150 mM Tris-HCl. Values are shown as percentages of the maximum activity. FTC-casein (▽) and FTC-RuBPCase (▼) were used as substrates.

Table II. *Effect of Various Compounds on the Activity of Tomato P-69 Proteinase*

The purified enzyme sample was preincubated with the compounds at the indicated concentration for 30 min in 4°C before the 1-h incubation with FTC-casein at pH 9.0.

Compound	Final Concentration	Activity Remaining
		%
None		100
Aprotonin	10 μg/ml	95
Pepstatin	10 μg/ml	99
Soybean trypsin inhibitor	10 μg/ml	97
leupeptin	1 mM	52.6
TPCK	1 mM	60.5
TLCK	1 mM	67.1
EDTA-Na_2	1 mM	78.4
1,10-Phenanthroline	1 mM	102.1
PMSF	1 mM	65
DTT	1 mM	148
pCMB	1 mM	20.2
pCMB	2 mM	0
$CaCl_2$	1 mM	171.6
$MgSO_4$	1 mM	145
$HgCl_2$	1 mM	41

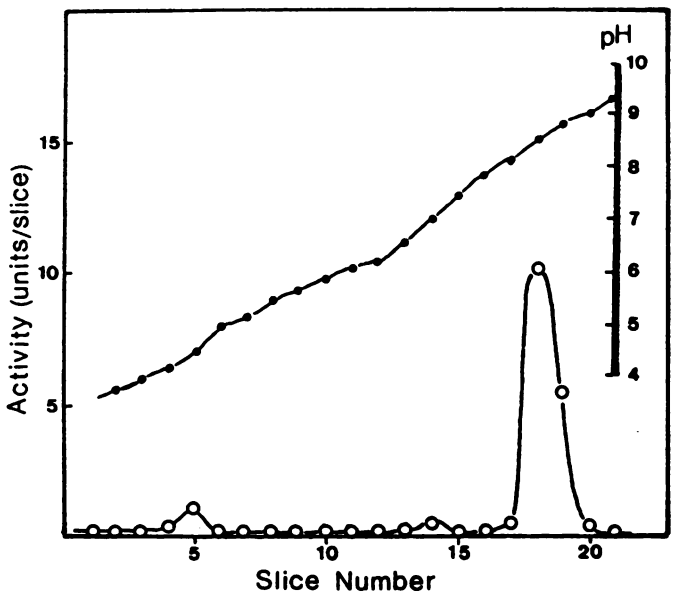

FIG. 7. Determination of enzyme activity after gel electrofocusing. The procedure was as described in "Materials and Methods." (○), Proteolytic activity; (●), pH of the different slices.

system (29). This behavior contrasts with that of PR proteins from tobacco and other species which are acidic proteins that can be separated with the Davis system (29).

The fact that proteinase P-69 has an alkaline optimal pH (8.5) suggests that this enzyme does not act at the level of the rather acidic lysosomal/vacuolar hydrolytic machinery, where many proteolytic systems have been described (2, 18, 24). Related to this the possibility of P-69 acting as a pathogenic protein in the chloroplast, causing part of the alterations associated with the disease, must be entertained. This would be compatible with its alkaline optimal pH and with the finding that the most dramatic cytopathic alterations after infection of tomato plants with viroids are the well pronounced disorganization and degradation of chloroplasts as well as general distortions in the cell wall (17, 21, 22). This hypothesis is validated in part by the finding that proteinase P-69 degrades RuBPCase (the most abundant protein of the chloroplast) large subunit, not at random but rather following a specific pattern. The well defined proteolytic fragments produced, many of which are subsequently cleaved into lower mol wt fragments, are stable against further digestion by proteinase P-69. The degradation of both subunits of RuBPCase followed different kinetics, the larger subunit being more rapidly degraded than the small subunit, which needed incubation times longer than 4 h to undergo detectable degradation (data not shown). This could be explained by the different mol wt of both subunits, in accord with the general scheme proposed for other proteolytic systems (1, 10). Unequivocal proof that the proteolytic system indicated above is involved in the loss of RuBPCase would be the detection of cleavage products of this protein. However, we

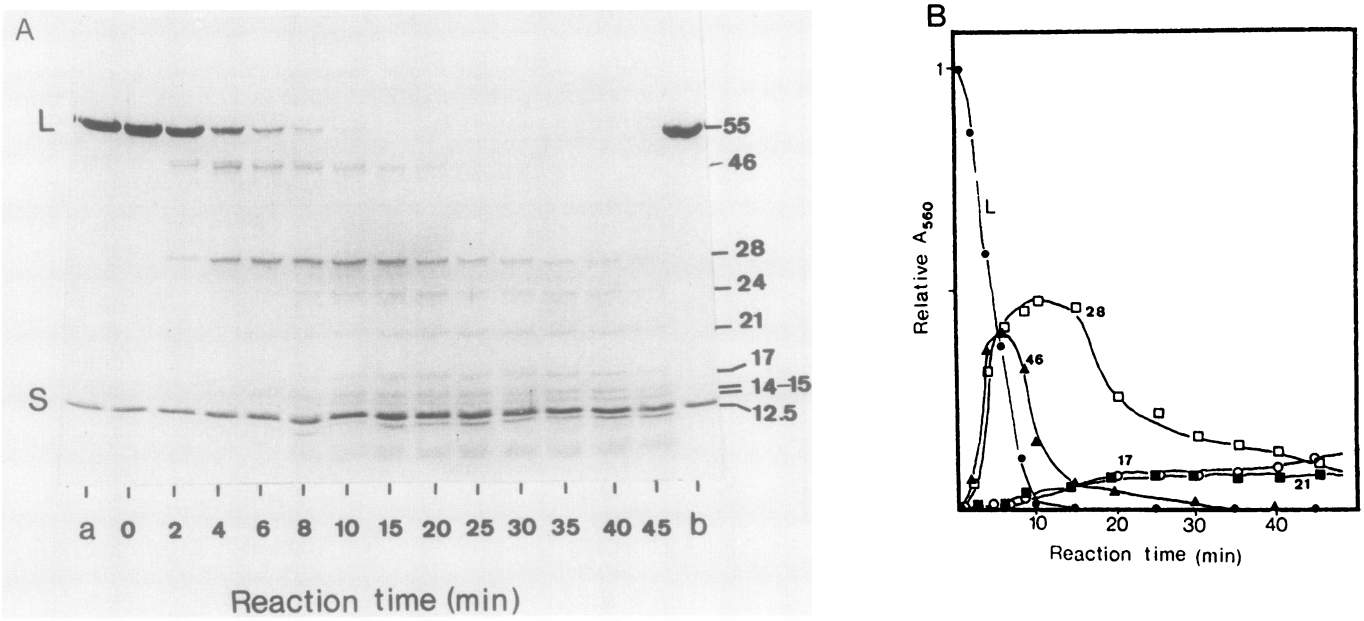

FIG. 8. Time course of the digestion of RuBPCase by proteinase P-69. A, Electrophoretic analysis of the different proteolytic fragments obtained after degradation of RuBPCase at the indicated times. Numbers on the right indicate the mol wt of the generated proteolytic fragments. L and S indicate the large and small subunits of RuBPCase, respectively. (a) and (b) denote a RuBPCase sample incubated for 0 and 45 min, respectively, in the absence of proteinase P-69. B, Densitometric analysis of the major proteolytic fragments shown in (A) expressed as relative A_{560}. Numbers denote the mol wt of the fragments scanned. L indicates the disappearance of RuBPCase large subunit.

have failed to detect such intermediates. As pointed out by Muller *et al.* (23), the failure to demonstrate intermediates in the degradation processes may be explained by the fact that they might be short-lived or unrecognizable by antibodies.

The hypothesis that proteinase P-69 is involved in the degenerative processes observed in the chloroplast does not necessarily require that the activity is located within the chloroplast. The enzyme may act during the last stages of chloroplast degradation, when its contents are released into the cytosol (17). Furthermore, in some host-pathogen systems, a decrease in the photosynthetic rate as well as a decrease in RuBPCase content occur upon infection (25). In this respect, P-69 proteinase increases in healthy plants as they age (data not shown), suggesting that P-69 is involved in normal aging of the leaves. Such involvement has been previously described for other PR proteins (6, 12).

A similar proteinase which has identical pH optimum, pI, and inhibitor selectivity, although with a different mol wt (37,000), has been reported in leaves of *Phaseolus vulgaris* by van der Wilden *et al.* (30). In this context it is interesting to note the finding of 'PM antigen' in tomato plants after infection with tomato plants macho viroid (TPMV) or potato spindle tuber viroid (PSTV) (11), and that a protein of similar mol wt accumulates in tomato leaves after inoculation with races of *Cladosproium fulvum* (9). If these proteins and P-69 were the same protein, we could conclude that proteinase P-69 is induced in tomato plants either as a degradative component of the response or as a potential component of defense.

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